UNIVERSIDADE FEDERAL DO PAMPA

CAMILA RAMÃO CONTESSA

BIOPLÁSTICO DE QUITOSANA/ÁGAR-ÁGAR INCORPORADO COM BACTERIOCINA PARA APLICAÇÃO COMO EMBALAGEM ATIVA

Bagé
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Dissertação apresentada ao programa de Pós-graduação em Ciência e Engenharia de Materiais da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciência e Engenharia de Materiais

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2021

Ficha catalográfica elaborada automaticamente com os dados fornecidos
pelo(a) autor(a) através do Módulo de Biblioteca do
Sistema GURI (Gestão Unificada de Recursos Institucionais) .

C183b Contessa, Camila Ramão
Bioplástico de quitosana/ágar-ágar incorporado com bacteriocina para aplicação como embalagem ativa / Camila Ramão Contessa.
74 p.

Tese(Doutorado)-- Universidade Federal do Pampa, MESTRADO EM CIÊNCIA E ENGENHARIA DE MATERIAIS, 2021.
"Orientação: Caroline Costa Moraes".

1. Polímero natural. 2. Lactobacillus sakei. 3. Bioconservante. 4. Creme de queijo minas frescal. I. Título.

DESPACHO

Nº do Processo: 23100.003242/2021-65
Interessado(s): GABRIELA SILVEIRA DA ROSA, CATARINA MOTTA DE MOURA

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Dissertação de Mestrado defendida e aprovada em: 22 de fevereiro de 2021.

Banca examinadora:

Assinado eletronicamente por **CAROLINE COSTA MORAES**, **PROFESSOR DO MAGISTERIO SUPERIOR**, em 08/03/2021, às 15:08, conforme horário oficial de Brasília, de acordo com as normativas legais aplicáveis.

Assinado eletronicamente por **CATARINA MOTTA DE MOURA**, **PROFESSOR DO MAGISTERIO**

SUPERIOR, em 08/03/2021, às 15:19, conforme horário oficial de Brasília, de acordo com as normativas legais aplicáveis.

Assinado eletronicamente por **Elessandra da Rosa Zavareze**, **Usuário Externo**, em 08/03/2021, às 15:22, conforme horário oficial de Brasília, de acordo com as normativas legais aplicáveis.

Assinado eletronicamente por **GABRIELA SILVEIRA DA ROSA**, **PROFESSOR DO MAGISTERIO SUPERIOR**, em 08/03/2021, às 15:22, conforme horário oficial de Brasília, de acordo com as normativas legais aplicáveis.

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Referência: Despacho do Processo nº 23100.003242/2021-65 SEI nº 0478376

RESUMO

Este trabalho visa o desenvolvimento de um filme bioplástico a partir de materiais naturais, com viés ambientalmente amigável que tenha por função conter, proteger e prolongar a vida útil de creme de queijo minas frescal. Foram utilizados dois polímeros naturais, quitosana e ágar-ágar, e glicerol como plastificante, sendo a formulação determinada utilizando a técnica de planejamento experimental, avaliando como respostas as características mecânicas dos filmes, essenciais para o manuseio do material. Após determinar a composição dos filmes e para garantir potencial ativo – propriedade antimicrobiana, adicionou-se extrato purificado de bacteriocina produzida por *Lactobacillus sakei* , isolado de uma matriz alimentar pelo grupo de pesquisa. A caracterização física, mecânica e microbiológica foi realizada para o filme com e sem extrato, para estudo da interação dos compostos presentes na matriz filmogênica. Os resultados obtidos indicam que a espessura variou entre 0,048±0,009 e 0,099±0,015 mm, a permeabilidade ao vapor de água entre 1,49 $x10^{-13}$±0,97 e 2,04$x10^{-13}$±0,28 $kg.m^{-1}.Pa^{-1}.s^{-1}$, tensão de ruptura entre 11,08±2,33 e 13,57±2,17 MPa, alongamento entre 15,51±2,87 e 18,66±2,44%, a solubilidade entre 10,28±1,038 a 20,97±2,958%, a propriedade de intumescimento entre 35,75±3,245 a 67,45±0,967%, o teor de água de 15,85±2,705 a 20,93±0,325% e a transmitância de 37,57±3,81 a 86,37±1,42% . A espectroscopia de infravermelho, análise de superfície e análise termogravimétrica constataram que houve a interação de todos os constituintes da solução filmogênica. A liberação do composto ativo foi gradual durante o tempo de contato com a solução simuladora, tendo entre o terceiro e o quinto dia os picos de liberação, permitindo, contudo, a redução do desenvolvimento microbiano presente durante todo o tempo analisado. As respostas antibacterianas *in vitro*, *in situ* e de microatmosfera evidenciaram a eficácia do filme ativo frente a microbiota teste, apresentando uma redução de 2,62 log UFC/g diretamente no alimento. Conclui-se que o filme possui capacidade ativa com características que favorecem a atuação como filme de barreira, e aumento da estabilidade microbiológica do alimento.

Palavras-Chave: Polímero natural. *Lactobacillus sakei*. Bioconservante. Creme de queijo minas frescal.

ABSTRACT

This work aims to develop a bioplastic film from natural materials, with an environmentally friendly bias and the function of containing, protecting and prolonging the lifespan of a perishable food product. Two natural polymers were used, chitosan and agar-agar, and a low molecular weight plasticizer. The formulation was determined using the experimental design technique, evaluating the mechanical characteristics of the films, which are essential for handling the material as responses. After determining the composition of the films and to ensure active potential, purified bacteriocina extract produced by *Lactobacillus sakei* and isolated from a food matrix by the research group was added. The physical, mechanical and microbiological characterization was performed for the film with and without extract, to study the interaction of the compounds present in the filmogenic matrix. The results obtained indicate that he thicknesses varied between 0.048±0.009 and 0.099±0.015 mm, the water vapor permeability between $1.49x10^{-13}$±0.97 and $2.04x10^{-13}$±0.28kg $m^{-1}Pa^{-1}s^{-1}$, ruptura stress between 11.08±2.33 and 13.57±2.17 MPa, elongation between 15.51±2.87 to 18.66±2.44%, the solubility between 10.28 ± 1.038 to 20.97 ± 2.958%, the swelling property between 35.75±3.245 to 67.45±0.967%, the water from 15.85±2.705 to 20.93±0.325% and transmittance from 37.57±3.81 to 86.37±1.42%. Infrared spectroscopy, surface analysis and thermogravimetric analysis found that all the constituents of the film-forming solution interacted. The release of the active compound was gradual during the time of contact with the simulator solution, having between the third and the fifth day the release peaks. The reduction of the microbial development showed effect during all the analyzed time. The in vitro, in situ and microatmosphere antibacterial responses showed the effectiveness of the active film against the test microbiota, showing a reduction of 2.62 log UFC / g directly in the food. It is concluded that the film has active capacity with characteristics that favor acting as a barrier film, and increasing the microbiological stability of the food.

Keywords: Natural polymer. *Lactobacillus sakei*. Bioconservative. Cream cheese minas frescal.

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LISTA DE ABREVIATURAS

ASTM - *American Society for Testing and Materials*

DTA - Doenças Transmitidas por Alimentos

EBA - *European Bioplastic Association*

FDA - *Food and Drug Administration*

GRAS - *Generally Recognized as Safe*

IFICF - *International Food Information Council Foundation*

NCCLS - *Nacional Committee for Clinical Laboratory Standards*

OMS - Organização Mundial da Saúde

PAE - Ésteres de Ácido Ftálico

PVC - Policloreto de Vinila

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CAPÍTULO 1

1 Introdução

Muitos materiais são usados na elaboração de diferentes tipos de embalagens, como vidro, metais, plásticos, madeira, dentre outros, além de combinações de mais de um material, como compósitos ou blendas. Quando não mais utilizados geram resíduos, alguns são destinados à reciclagem e a grande maioria destinados aos aterros municipais, gerando preocupação ambiental devido ao tempo que levam para se decompor (JIMÉNEZ-ROSADO *et al*., 2019).

Com o crescente desenvolvimento populacional, a busca por novos produtos e tecnologia avançada, tem gerado a necessidade de uma grande produção de embalagens a fim de conter estes produtos. Como consequência, tem-se uma geração exorbitante de resíduos, principalmente resíduos sólidos, que em sua maioria demoram centenas e milhares de anos para se decompor no meio ambiente, acarretando não somente uma crise ambiental, como também problemas econômicos e sociais (RAHMANI *et al.*, 2013; GANESHI, 2016).

Estima-se que em torno de 300 milhões de toneladas de embalagens plásticas são depositadas nos depósitos de lixo anualmente (ORGANIZAÇÃO DAS NAÇÕES UNIDAS, 2019). Além da superlotação causam a morte de centenas de animais que acabam por consumi-las, sendo que a indústria alimentícia, é o setor que mais contribui com a geração de resíduos, pois representam em torno de 50% em peso do total de embalagens vendidas (MARSH; BUGUSU, 2007; RAMOS, 2019).

As embalagens biodegradáveis ativas incorporadas de antimicrobianos têm se destacado muito nos últimos anos, uma vez que sua utilização pode ser eficiente na redução e/ou inibição do desenvolvimento microbiano (LATOS-BROZIO; MAZEK, 2020; ZHAO *et al*., 2019; KHAN *et al*., 2017). A utilização de bacteriocinas se torna interessante, uma vez que são de natureza proteica e apresentam potencial bioativo, possuem ação antimicrobiana com ação específica, e um espectro antimicrobiano amplo, atuando na inibição de bactérias patogênicas e deteriorante de alimentos (ZOU *et al*., 2018).

Aliado ao aumento da vida útil de produtos alimentícios se tem a redução na geração de resíduos, uma vez que a taxa de degradação de embalagens desenvolvidas com materiais biodegradáveis é muito maior quando comparada as elaboradas com materiais convencionais (PEELMAN *et al.,* 2013). Segundo uma pesquisa realizada pela *International Food Information Council Foundation* (IFICF) atualmente o consumidor está mais consciente com o meio ambiente, sendo a produção sustentável um dos principais fatores na escolha de um

produto alimentício (INTERNATIONAL FOOD INFORMATION COUNCIL FOUNDATION, 2018).

Desta forma a equipe do Laboratório de Microbiologia e Toxicologia de Alimentos e o Grupo de Pesquisa de Engenharia de Processos em Sistemas Particulados da Universidade Federal do Pampa, Campus – Bagé RS, desenvolvem projetos nesta temática, investigando e sintetizando novos materiais que apresentem características e propriedades adequadas para utilização no prolongamento da vida útil de alimentos perecíveis, onde já desenvolveram várias pesquisas inclusive o depósito de patentes.

CAPÍTULO 2

1 Objetivo geral

O objetivo desta pesquisa foi desenvolver filmes bioplásticos ativos à base de blenda polimérica composta por quitosana e ágar-ágar, incorporado de extrato de bacteriocina como antibacteriano, com características adequadas para conter, proteger e aumentar a vida útil de creme de queijo minas frescal.

2 Objetivos específicos

- Estudo da formulação do bioplástico para determinar o efeito da concentração de biopolímeros (quitosana e ágar-ágar) e de plastificante nas propriedades mecânicas do filme;
- Otimização das condições de formulação do filme e caracterização física, mecânica, de barreira e microbiológica;
- Produção, extração, purificação e caracterização microbiológica de antibacteriano natural composto de bacteriocina obtida de *Lactobacillus sakei*;
- Formulação e caracterização de filme composto por blenda polimérica, plastificante e incorporado com extrato de bacteriocina;
- Aplicação *in situ* do filme bioplástico em creme de queijo minas frescal e acompanhamento da estabilidade microbiológica.

CAPÍTULO 3

1 Revisão da literatura

1.1Embalagens alimentares

Embalagens de alimentos têm por objetivo permitir o transporte, distribuição e manuseio, garantindo proteção contra choques e compressões, além de minimizar as perdas do produto por deterioração através do controle de umidade, oxigênio, luz e barreira contra micro-organismos, atuando como barreira a atmosfera envolvente (ROBERTSON, 1992).

Sendo assim a embalagem deve ter desempenho compatível com a sua funcionalidade, atendendo as quatro funções básicas (proteção, comunicação, conveniência e contenção) atendendo as características de cada produto e processamento que será submetida, necessitando de uma boa resistência mecânica, flexibilidade e elasticidade a fim de evitar rasgos e perfurações durante todas as etapas de produção, estocagem e comercialização do produto, além de ser um veículo de comunicação entre o produtor e o consumidor, através do *marketing* (GHAANI *et al*., 2016 ; HAN, 2005).

As embalagens são consideradas um veículo de venda e divulgação da marca, uma vez que é o primeiro contato do consumidor com o produto, tornando-se uma das principais características para a decisão na hora da compra (DELLA LUCIA *et al*., 2007). A embalagem deve ainda ser composta por material inerte, para que não haja migração de seus compostos para o alimento, para que assim não cause risco à saúde do consumidor e/ou altere as características sensoriais do mesmo (LATOS-BROZIO; MAZEK, 2020).

A grande maioria de embalagens alimentícias é originária de polímeros de origem petroquímica devido as suas características de flexibilidade e leveza, contudo é uma fonte não renovável. Desta forma a sua utilização resulta em problemas socioeconômicos como o aumento no preço do petróleo, assim como, geração e acúmulo de resíduos que podem levar dezenas ou centenas de anos para se decompor na natureza (WARING, HARRIS, MITCHELL, 2018).

No ano de 2012 o mercado de embalagens de alimentos contribuiu com uma participação estimada de 69% do consumo de embalagens de todos os setores. Em 2015 correspondeu a 73% diante do mercado global de embalagens. Em 2018 somente na União Europeia 1.130 bilhões de embalagens de alimentos e bebidas foram destinadas aos aterros sanitários (KETELSEN; JANSSEN; HAMM, 2020).

A produção de resíduos sólidos oriundos de embalagens alimentícias cresce a uma taxa de 4,2% ao ano desde 2010, e estima-se que até 2024 esse valor se mantenha linearmente. Considerando todos os materiais utilizados no desenvolvimento destas embalagens o plástico corresponde a maior participação, com 47% do total de embalagens do setor em 2015 (ALL4PACK, 2016).

Plásticos são polímeros, formados pela união de várias unidades menores, denominadas monômeros, os tipos mais comuns são o polietileno, de alta e baixa densidade, polipropileno e poliestireno. Dentre a grande quantidade de materiais plásticos produzidos, 40% são embalagens de uso único, e em sua maioria não são biodegradáveis, acarretando na poluição atmosférica, aquática e terrestre (WARING, HARRIS, MITCHELL, 2018).

O consumo de filme plástico, aumentou muito nos últimos anos e aliado ao longo período de decomposição se tem a utilização de ésteres de ácido ftálico (PAEs) utilizado como plastificante na elaboração de filmes plásticos de policloreto de vinila (PVC) e que possui efeito carcinogênico a seres vivos (LI *et al.,* 2020). Comumente os filmes mais utilizados diariamente são os filmes plásticos de PVC, e diante desta problemática tem-se buscado meios alternativos para reduzir tais impactos, através do desenvolvimento de bioplásticos, bem como a busca por novas tecnologias (PEELMAN *et al*., 2013).

1.2 Bioplásticos

De acordo com a Associação Europeia de Bioplásticos (*European Bioplastic Association* – EBA), são definidos como plásticos a base de recursos renováveis, biodegradáveis ou ainda baseados em materiais biológicos. Estes materiais se decompõem na presença de dióxido de carbono, metano, água e biomassa, através da ação enzimática de micro-organismos, sendo capazes de se decompor na mesma taxa de outros materiais compostáveis conhecidos. A etapa inicial de compostagem se dá por processo abiótico, ou seja, por condição térmica, sendo que os fragmentos oriundos dessa etapa da decomposição devem ser completamente utilizados por micro-organismos (SONG *et al*., 2009).

A indústria de embalagens de alimentos busca alternativas biodegradáveis a fim de melhorar sua sustentabilidade, desta forma vem investindo nestes materiais bioplásticos, onde atualmente vários estudos estão focados, principalmente em técnicas de síntese. Contudo diante de toda uma investigação, algumas questões ainda são falhas como o uso em larga escala e algumas propriedades que ainda são restritas (ZHAO *et al*., 2019).

Algumas das limitações no que se refere às propriedades do material estão relacionadas com a fragilidade, instabilidade térmica, baixa resistência ao impacto, alta permeabilidade ao vapor de água e oxigênio, que ao serem utilizadas em alimentos frescos, o mesmo fica suscetível a perda de umidade alterando as propriedades sensoriais do produto (KHAN *et al*., 2017; CYRAS; SOLEDAD; ANALÍA, 2009).Contudo é nesse âmbito em que os estudos estão voltados atualmente, baseando-se em investigar estratégias para melhorar suas propriedades a fim destes novos materiais poderem resistir aos possíveis tratamentos da indústria alimentícia e também possam conter o alimento com suas propriedades sensoriais por mais tempo (ZHAO *et al*., 2019).

Dentre os diversos materiais utilizados na síntese de filmes bioplásticos, tem-se amido, celulose, gomas, quitosana e pectinas, dentre os quais cada qual apresenta uma propriedade especifica na elaboração de bioplásticos (LOPEZ *et al*., 2015). No geral bioplásticos a base de polissacarídeos como amido, quitosana e carragena possuem limitações nas propriedades mecânicas, contudo possuem uma baixa permeabilidade a gases (KJELLGREN *et al*., 2006), quando a base é de proteína como caseína e colágeno as propriedades mecânicas são aceitáveis enquanto são carentes nas características físicas (LIMPAN *et al*., 2010). Já os bioplásticos a base de lipídeos, possuem uma ótima barreira a umidade, contudo possuem sensibilidade à oxidação (WANG *et al*., 2014).

Por esse motivo se tem pesquisado por misturas destes biopolímeros na elaboração de filmes bioplásticos, de forma a se ter um melhoramento nas características finais, podendo apresentar uma ampla gama de estruturas com diferentes propriedades, permitindo assim direcionar as características para a aplicação desejada (JIMÉNEZ-ROSADO *et al*., 2019**).** A incorporação de aditivos como plastificantes auxiliam no melhoramento das características finais, são moléculas de baixo peso molecular que atuam na modificação da estrutura tridimensional, reduzindo as forças intermoleculares, aumentando a mobilidade das cadeias poliméricas e diminuindo a permeabilidade a gases (MATVEEV; GRINBERG; TOLSTOGUZOV, 2000).

1.2.1 Ágar-Ágar

Ágar-ágar é um biopolímero pertencente aos polissacarídeos naturais extraídos de algas vermelhas, da classe *Rhodophyta*, sendo o carboidrato estrutural da parede dessas células. É composto por agarose, que possui uma cadeia linear, e agaropectina que possui uma cadeia ramificada, ligadas entre si por ligações α- (1 → 3) e β- (1 → 4) (PERVEZ *et al*., 2019).

É um material termoplástico, biocompatível e biodegradável além de ser muito abundante na natureza, e se torna atraente devido a sua estrutura química, resistência a ácido e capacidade de formar um gel consistente mesmo que em baixas concentrações, favorecendo desta forma a aplicação em diversos ramos industriais (CLARK; ROSS-MURPHY, 2005).

Diante das diversas aplicações, destaca-se o setor alimentício, utilizado como espessante e também em embalagens alimentares (HUANG *et al*., 2020), na agricultura atuando como condicionadores de solo e absorvedores de água, muito eficientes em locais com pouca disponibilidade de ágar-ágar (HASIJA *et al*., 2018), em medicamentos, na microencapsulação de medicamentos e compostos bioativos (KAVOOSI *et al*., 2018). Além de possuir uma matriz altamente porosa, sendo interessante para o aprisionamento de partículas (PERVEZ *et al*., 2019).

Filmes a base deste polímero, no entanto, são de natureza quebradiça, possuem fracas propriedades mecânicas e alta sensibilidade a água, pois são de natureza hidrofílica, limitando desta forma sua aplicação em um produto de alta umidade (SHANKAR; REDDY; RHIM, 2015). No entanto existem alguns estudos que utilizam blendas ou reforço deste polímero para um resultado final com diferentes características, sejam essas químicas, físicas ou mecânicas. Jridi *et al*. (2019) utilizaram a união de gelatina e ágar-ágar, resultando assim em filmes mecanicamente mais resistentes. Phanwipa; Harnkarnsujarit (2020) obtiveram melhorias na solubilidade do filme, quando desenvolvido utilizando misturas de amido, ágar-ágar e maltodextrina. Arfat, Ahmed e Jacob (2017) utilizaram de nanopartículas de liga bimetálica (Ag-Cu) em filmes a base de ágar-ágar para aumentar a propriedade de barreira ao oxigênio.

1.2.2 Quitosana

A quitosana é derivada da quitina e foi descoberta em 1859, quando Rouget cozinhou quitina, descoberta em 1811, em hidróxido de potássio e descobriu que se tornou solúvel em ácidos orgânicos (ROUGET, 1859; BAKSHI *et al*., 2018). A quitina é o segundo polímero natural mais abundante na natureza e é obtida principalmente de resíduos de cascas de caranguejo e camarão, geralmente utilizados de industrias de frutos do mar, contudo pode ser obtida de diversas outras fontes como, conchas de moluscos, parede celular e membrana celular de fungos, componente da parede celular de algas, exoesqueleto de insetos e aracnídeos (BAKSHI *et al*., 2018).

A desacetilação da quitina se dá a partir da transformação da acetamida ($NHCO_3$) em amina (NH_2) ocorrido em meio básico, é produzida a partir de diferentes graus de desacetilação e pesos moleculares que variam desde a concentração alcalina, tempo e temperatura utilizados no processo (NO *et al*., 2007).

A quitosana é o único polissacarídeo de natureza alcalino, os outros são de origem ácida ou neutros, é um composto atóxico, biocompatível e biodegradável e é absorvida pelo corpo (DINESH; SINGH; RAY, 2007). As propriedades da quitosana estão diretamente ligadas ao peso molecular, grau de desacetilação e grau de cristalinidade. Propriedades como viscosidade, solubilidade, resistência à tração e alongamento são influenciadas pelo peso molecular, que corresponde ao número de unidades de açúcar por molécula do polímero, a viscosidade da solução de quitosana é aumentada com o aumento do grau de desacetilação (KLOSSNER *et al*., 2008).

Polímeros de quitosana são aminopolissacarídeos com estruturas únicas, possuindo várias propriedades, e uma alta funcionalidade, podendo ser aplicada as mais diversas áreas, tanto industrial como área biomédica (DASH *et al*., 2011) é um dos polímeros de origem biológica mais promissor, podendo ser utilizado como aditivo alimentar na dieta (RAMYA *et al*., 2012), utilizada em medicamentos, pois tem um grande potencial em atuar como antiácido, protegendo o estomago de outras drogas além de atuar como transportador e liberador de drogas no corpo humano (BALDRICK, 2010), em cosméticos, no tratamento de cabelos e pele, pois atua como agente hidratante e possui a capacidade de adesão a fragrância (TZANEVA *et al*., 2017), teve propriedades antivirais relatadas (MORIMOTO *et al*., 2001) além de atuar como agente antimicrobiano, atuando na superfície externa de bactérias, como a parede celular de micro-organismos Gram-negativos compostos por lipopolissacarídeos e no peptidoglicano associado ao ácido teicóico associado a membrana celular de Gram-positivos (GOY; BRITTO; ASSIS, 2009).

No entanto, a quitosana possui algumas desvantagens para aplicações em filmes bioplásticos quando utilizada como única fonte de polímero, pois apresenta baixasolubilidade, não permitindo a interação com outros compostos muitas vezes utilizados para a elaboração de um filme, como o plastificante, desta forma a união deste com outros polímeros permite um filme com excelentes características (BAKSHI *et al*., 2018). Ghaderi *et al*., (2019) obtiveram melhoramento nas propriedades de barreira e solubilidade de filmes a base de quitosana e álcool vinílico quando adicionado gelatina de peixe. Mendes *et al*., (2016) produziram filmes com melhor extensibilidade e estabilidade térmica ao utilizar mistura de quitosana e amido de milho.

1.3 Embalagens bioplásticas ativas

Embalagens ativas visam melhorar as características do alimento, ultrapassando o papel passivo de proteção, pois são capazes de modificar as condições do produto de forma a prolongar sua vida útil, mantendo suas propriedades sensoriais e de segurança. Suas funções adicionais são divididas em absorção de compostos e liberação de compostos. Os sistemas absorvedores contribuem com a remoção de compostos indesejáveis responsáveis pela aceleração da degradação do alimento, como o oxigênio, excesso de água, etileno, dióxido de carbono dentre outros. Já os emissores, tem por função a liberação de compostos, os quais auxiliam no prolongamento da vida útil, sendo esses, dióxido de carbono, etanol, antioxidantes ou antimicrobianos (AHVENAINEN, 2003).

Embalagens ativas incorporadas com antimicrobianos têm se destacado muito nos últimos anos, uma vez que as reações de deterioração têm início na superfície do alimento, sendo assim a utilização de um filme de recobrimento é eficiente na redução e/ou inibição do desenvolvimento microbiano (BERNARDI; GARCIA, COPETTI, 2019). A incorporação desses agentes tem por vantagem a redução no teor de conservantes utilizados no alimento, atendendo o consumidor que busca por alimentos com teores mínimos de aditivos (ZANETTI *et al*., 2018).

Para que uma embalagem antimicrobiana seja eficiente é essencial que fique em contato direto com o alimento, de forma que haja migração do composto presente na embalagem para a superfície do produto, quando utilizado composto com propriedades de volatilidade o contato direto da embalagem-produto não se faz necessário (VERMEIREN; DEVLIEGHERE; DEBEVERE, 2002).

O desenvolvimento de filmes biodegradáveis para aplicação em produtos alimentícios incorporados de extratos com propriedades antioxidantes e antimicrobianas tem motivado vários grupos de pesquisas (YOUNG *et al*., 2019), que na grande maioria tem buscado por compostos de origem vegetal. O Quadro 1 apresenta alguns estudos que vêm sendo desenvolvidos com a utilização destes compostos. Os extratos de plantas recebem grande foco devido as altas concentrações de compostos fenólicos que conferem elevada atividade antioxidante (KANMAMI; RHIM, 2014).

Quadro 1 - Filmes bioplásticos incorporados de diferentes extratos

Matriz polimérica	**Extrato**	**Ação**	**Referência**
Quitosana	Batata doce de polpa roxa	Antioxidante	YOUNG *et al*. (2019)
Amido	Chá verde e manjericão	Antioxidante	MEDINA-JARAMILLO *et al.* (2017)
Quitosana	Casca de semente de soja preta	Antioxidante	WANG *et al*. (2019a)
Policaprolactona e quitosana	Semente de toranja	Antimicrobiano	WANG *et al*. (2019b)
Ágar-ágar e gelatina	Chá verde	Antioxidante e Antimicrobiano	Giménez *et al*. (2013)
Quitosana	Mirtilo e amora	Antioxidante	KUREK *et al*. (2018)
Gelatina e ágar-ágar	Folhas de videira	Antioxidante	JRIDI *et al.* (2019)
Quitosana	Casca de maça	Antioxidante e Antimicrobiano	RIAZ *et al*. (2018)
Quitosana e nanopartículas de TiO_2	Casca de ameixa preta	Antioxidante e antimicrobiana	ZHANG *et al*. (2019)

Fonte: Autora (2021)

A adição de extratos em filmes resulta em impactos nas propriedades físico, químicas, mecânicas, de barreira, antioxidantes e antimicrobianas.Com base nisso tem sido utilizada em uma ampla variedade de funções, observando que esses compostos não atuam somente como antimicrobianos ou antioxidantes, mas também modificam as propriedades da embalagem, melhorando sua aplicação no geral (MIR *et al*., 2018).

A utilização de extratos naturais tem como principal objetivo agregar os compostos ativos ao produto alimentício. Com tudo, inúmeras pesquisas utilizam compostos de origem não renováveis como as nanopartículas de metais a fim de atingir características antimicrobianas eficientes.

Xu *et al*. (2018) incorporaram nanopartículas de prata a filmes de quitosana para o desenvolvimento de uma embalagem com atividade antimicrobiana. Peighambardoust*et al*. (2019) desenvolveram filmes ativos a base de amido incorporados com uma combinação de nanopartículas de Ag, ZnO e CuO para potencial uso como embalagem de alimentos. Zhixiang *et al.* (2019) desenvolveram filme antimicrobiano a base de *curdlan* (goma extraída de uma bactéria do sistema digestivo humano, utilizado como espessante na indústria alimentícia) e nanopartículas de prata sintetizadas com *Glycyrrhiz* (planta com propriedade medicinal).

A fim de elaborar um filme bioplástico com compostos naturais e com potencialidade para aplicação em embalagens ativas com ação antimicrobiana que seja eficiente, tem-se o estudo deste trabalho, que utiliza extrato purificado de uma bacteriocina produzida por bactéria ácido lática extraída de uma matriz alimentícia.

1.4 Bacteriocinas

Bacteriocinas são compostos de natureza proteica sintetizadas no ribossomo e liberadas para o meio extracelular, são biologicamente ativas possuindo ação bacteriostática ou bactericida sob bactérias incluindo as patogênicas, são produzidas por bactérias ácido láticas, as quais possuem status GRAS (*Generally Recognized as Safe*) (OGAKI; FURLANETO; MAIA, 2015).

Em geral, são pequenas proteínas apresentando de 20-60 aminoácidos, catiônicas, hidrofóbicas, possuem ponto isoelétrico elevado e variam quanto ao espectro de ação conforme o micro-organismo produtor, propriedades bioquímicas e/ou peso molecular, sendo que em sua maioria apresentam baixa massa molecular de 3 a 10 kDa, onde são divididas de acordo com suas características físico-químicas e propriedades estruturais, apresentando-se em 4 classes, sendo que as classes I e II são conhecidas por apresentarem termoestabilidade e peso molecular inferior a 10 kDa, as classes III e IV apresentam peso molecular acima de 30 kDa e são termolábeis (MARTINIS; ALVES; FRANCO, 2002).

Seu efeito bactericida ou bacteriostático se dá pela ação na parede celular do organismo alvo, inibindo a biossíntese da parede celular ou acarretando na formação de poros na mesma, resultando na morte celular. A formação de poros ocorre em duas fases, no primeiro momento há o envolvimento das cargas positivas e resíduos polares da bacteriocina com os fosfolipídios de carga negativa, presentes na camada lipídica da membrana alvo, neste momento a bacteriocina se torna sensível ao ataque de enzimas proteolíticas. Contudo no segundo momento se tem a formação dos poros nas células alvo, ocorrendo desta forma a morte da célula (BRUNO; MONTVILLE, 1993).

A biossíntese destes peptídeos biologicamente ativos é afetada por várias condições ambientais como pH, temperatura, aeração, capacidade tamponante do meio e tempo de incubação da bactéria produtora. Essa produção acontece ao final da fase log e início da fase estacionária do crescimento bacteriano, onde são excretadas então para o meio, podendo permanecer aderidas na célula de algumas bactérias (KUMARIYA *et al*., 2019).

A utilização de bacteriocinas na indústria de alimentos é uma forma alternativa para o aumento da segurança e qualidade do alimento e na substituição de conservantes químicos (MORENO; LERAYER; LEITÃO, 1999). São produzidas por micro-organismos considerados como seguros ou de presunção qualitativa de segurança, e geralmente são digeridas pelo organismo humano, não havendo índices de intoxicação e patogenicidade (O'CONNOR *et al*., 2020), dessa forma tem sido foco em várias pesquisas que preconizam alimentos livres de aditivos químicos (SILVA; SILVA; RIBEIRO, 2018).

No que se refere a embalagens ativas, a incorporação de bacteriocinas é muito restrita, havendo estudos de aplicação de bacteriocinas comercias, como a nisina e a pediocina, devido à sua aprovação GRAS pela *Food and Drug Administration* (FDA) dos EUA (SOBRINO-LÓPEZ; MARTÍN-BELLOSO, 2008). E mesmo assim são muito isolados, Narsaiah *et al*., (2015) estudaram a vida útil de mamão minimamente processado com revestimento de alginato incorporado de pediocina. Meira *et al*., (2017) produziram filmes com matriz polimérica de

amido milho, incorporados de nisina e pediocina. Santiago-silva *et al*., (2009) avaliaram o efeito antimicrobiano de filmes com matriz de celulose e incorporados com pediocina na preservação de presunto fatiado. Xiong *et al*. (2020) avaliaram o armazenamento de carne de porco fresca com revestimento comestível a base de quitosana-gelatina incorporados de extrato de nisina com semente de uva.

A nisina e pediocina apresentam atividade antimicrobiana contra uma ampla variedade de bactérias Gram-positivas, havendo relatos de utilização como bioconservantes naturais em alimentos à base de carne e produtos lácteos, contudo seu espectro contra micro-organismos Gram-negativos é deficiente (DEEGAN *et al*., 2006). Dessa forma a investigação por bacteriocinas com amplo espectro de ação, tanto para micro-organismos Gram-positivos quanto Gram-negativos se torna promissor.

1.5 Impacto da formulação dos bioplásticos em suas propriedades

A compatibilidade da mistura de dois ou mais materiais é um grande desafio, porém quando se consegue essa interação, seja entre polímeros de origem petroquímica ou de origem biológica, como os bioplásticos, apresentam um alto potencial de aplicação, pois a interação destes compostos possibilita uma gama de características físico-químicas, mecânicas e de barreira (PEELMAN *et al*., 2013). Quando se utiliza ainda outros compostos, como os extratos com propriedades ativas a possibilidade de alteração dessas características são maiores, devido as diferentes ligações dos compostos com a matriz polimérica e da própria matriz entre si (WANG *et al.,* 2012).

Mir *et al*. (2018) descrevem algumas dessas alterações, principalmente nas propriedades de espessura, permeabilidade ao vapor de água , resistência à tração , solubilidade e propriedades de barreira.

A espessura é um parâmetro que influencia diretamente nas propriedades físicas, mecânicas e de barreiras do filme, geralmente a adição de diferentes extratos proporciona obtenção de filme mais espesso, devido ao aumento no conteúdo sólido adicionado e em algumas vezes altera a cristalinidade da estrutura polimérica (DELGADO *et al*., 2018).

As propriedades mecânicas como resistência à tração e alongamento na ruptura, são muito importantes nas propriedades do filme, pois permite que tenham resistência adequada, mantendo a integridade dos produtos embalados durante o transporte, manuseio e armazenamento. A resistência à tração é a força máxima que o filme resiste antes do rompimento e o alongamento é a flexibilidade máxima do filme antes do rompimento. A adição

de extratos naturais influencia essas propriedades devido à interação com que se ligam, sendo assim a origem do extrato, e da matriz polimérica interferem, adquirindo diferentes combinações (NORAJIT; KIM; RYU, 2010). Tan *et al*. (2015) ao adicionarem extrato de semente de toranja, obtiveram filmes mais amorfos e com menor resistência a tração, já Siripatrawan e Harte (2010) obtiveram um aumento na resistência à tração e alongamento ao adicionarem chá verde na matriz de quitosana, contudo na matriz de ágar-gelatina as propriedades foram reduzidas.

As propriedades de barreira são uma das propriedades mais importantes para aplicação em alimentos, uma vez que determina a vida útil do produto, pois permite ou não a permeabilidade do vapor de água, essa propriedade depende da estrutura morfológica do filme, quanto mais compacto, menor a permeabilidade, e aumenta a propriedade de barreira, extratos quando adicionados alteram significativamente essas propriedades (BIFANI *et al*., 2007).

Estudos com foco no melhoramento das propriedades dos bioplásticos através de alterações na formulação têm sido desenvolvidos (WANG *et al*., 2018). O Quadro 2 apresenta alguns estudos do melhoramento dessas matrizes poliméricas.

Quadro 2 - Melhoramento de matrizes poliméricas.

Matriz	Melhoramento	Propriedade influenciada	Referência
Amido de milho	Diferentes concentrações de quitosana	Aumento na resistência à tração e alongamento na ruptura	Ren *et al*. (2017)
Gelatina	Quitosana	Aumento nas propriedades mecânicas e diminuição na permeabilidade ao vapor de água	Hosseini *et al*. (2013)
Ágar-ágar	Nanopartículas de liga bimetálica (Ag-Cu)	Melhoramento termomecânico e de barreira ao O_2	Arfat; Ahmed; Jacob (2017)
Ágar-gelatina	Nanopartículas de TiO_2	Diminuiu a permeabilidade ao vapor de água, aumento da resistência à tração e aumento da propriedade de barreira a luz UV	Vejdan *et al*. (2016)
Ágar-ágar	Nanocompósitos de quitosana e halloysites combinados	Aumento na resistência à tração e diminuição no grau de intumescimento	Huang *et al*. (2020)
Ágar-ágar	Celulose nanobacteriana	Aumento da estabilidade térmica e propriedades mecânicas	Wang*et al*. (2018)
Quitosana	ZnO	Reduziu a propriedade de intumescimento	Al-Naamani; Dobretsov; Dutta, (2016)
Quitosana-gelatina	Nanocompósitos de Ag	Diminuição da transmitância da luz	Kumar *et al*. (2018)

Fonte: Autora (2021)

Em geral a união de dois ou mais biopolímeros, ou até mesmo a inserção de nanopartículas metálicas contribui de forma positiva com as propriedades dos filmes, desta forma a investigação deve ser aprofundada na combinação da matriz polimérica e dos demais componentes adicionados à formulação do filme, pois cada combinação resulta em propriedades distintas (MALAFAYA; SILVA; REIS, 2007).

CAPÍTULO 4

Artigo 1: Artigo aceito para publicação na International Journal of Molecular Sciences, ISSN:1422-0067, qualis A2 na área de materiais, fator de impacto 4,556

CHITOSAN/AGAR-AGAR-BASED BIOPLASTIC FILM INCORPORATED OF BACTERIOCINE FOR APPLICATION IN CREAM CHEESE MINAS FRESCAL

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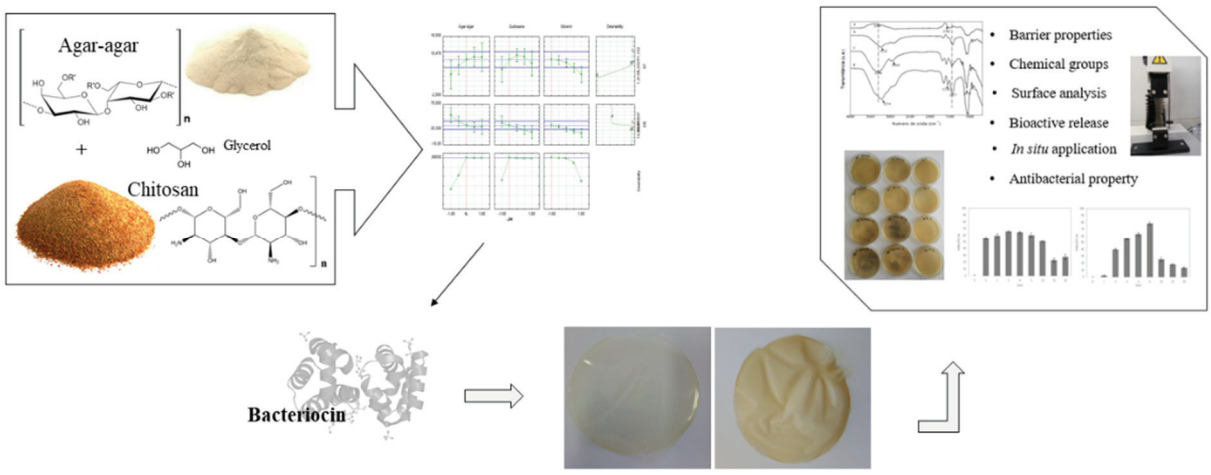
Highlights

- Chitosan / agar films were prepared with purified extract of a bacteriocin obtained from a food matrix.
- The films presented low water solubility and water vapor permeability, a good alternative for food barrier.
- The opacity increased with the addition of bacteriocin extract and the mechanical characteristics were unchanged.
- The chitosan/agar-agar composite films incorporated of bacteriocin showed bacterial activity under food-borne pathogens.
- The film has an active capacity, as it has increased the microbiological stability of the minas frescal cheese.

Graphical Abstract



ABSTRACT

The objective of this work was to develop a chitosan/agar-agar bioplastic film incorporated with bacteriocin that presents active potential when used as food packaging. The formulation of the film solution was determined from an experimental design, through the optimization using the desirability function. After establishing the concentrations of the biopolymers and the plasticizer, the purified bacteriocin extract of *Lactobacillus sakei* was added, which acts as an antibacterial agent. The films were characterized through physical, chemical, mechanical, barrier and microbiological analyses. The mechanical properties and water vapor permeability were not altered by the addition of the extract. Swelling showed a decresing and solubility an increasing.. Visible light protection was improved by increased opacity and antibacterial capacity was effective. When used as cream cheese minas frescal packaging it contributed to the increase of microbiological stability, showing a reduction of 2.62 log UFC / g, contributing a gradual release of the active compound into the food during the storage time. The film had an active capacity that could be used as a barrier to the food, allowing it to be safely packaged.

Keywords: Active packaging, barrier property, *Lactobacillus sakei*.

1 INTRODUCTION

Bioplastics have attracted the attention of researchers as a possible alternative to plastic products from petrochemical and non-renewable sources, which are responsible for several environmental problems, especially associated with difficult degradation (Albuquerque & Malafaia, 2018). These alternative and ecologically correct materials are important for sustainable growth because they are produced from renewable resources (Brodin, Vallejos, Opedal, Area & Chinga-Carrasco, 2017).

Many biopolymers have been used in the elaboration of bioplastics. Agar-agar is a biopolymer belonging to the natural polysaccharides extracted from red algae, of the *Rhodophyta* class, being the structural carbohydrate of the wall of these cells (Pervez et al., 2019). It is a thermoplastic, biocompatible and biodegradable material, widely used in several industrial branches. Bioplastic films based on this polymer, however are brittle in nature, have poor mechanical properties and high sensitivity to water, because they are hydrophilic in nature, thus limiting its application in a product of high humidity (Shankar; Reddy & Rhim, 2015).

Chitosan is derived from chitin, obtained mainly from residues of crab and shrimp shells, however it can be obtained from several other sources such as cell wall and cell membrane of fungi, exoskeleton of insects and arachnids (Bakshi, Selvakumar, Kadirvelu & Kumar, 2018). The deacetylation of chitin occurs from the transformation of acetamide ($NHCO_3$) into amine (NH_2) in basic medium. They can be produced with different degrees of deacetylation and molecular weights, which directly influence the properties of bioplastics, such as viscosity, solubility, tensile strength and elongation (Klossner, Queen, Coughlin, & Krause, 2008; No, Meyers & Xu, 2007). Chitosan has some disadvantages for applications in bioplastic films because it presents low solubility, not allowing the interaction with other compounds often used for the elaboration of a film (Bakshi, Selvakumar, Kadirvelu & Kumar, 2018).

In this way the union of these two polymers allows the synthesis of a bioplastic film with appropriate characteristics for application in the food industry, since the hydrophobicity of chitosan allows the application in products with high water activity, and the thermal characteristics of agar-agar allow the integrity of the material in relation to the temperatures used in the sterilization processes, for example (Phan, Debeaufort, Voilley, & Luu, 2009; Setareh, & Hosseini, 2017).

Research has been developing new materials that aim to expand the potential for the application of bioplastics in the packaging industry. An alternative is the addition of active compounds in the biopolymeric matrix. The bacteriocin extract has as main objective to add antimicrobial characteristics and contribute to the synthesis of a material that can be applied in a food product, acting in the protection, storage and prolongation of the useful life. The use of bacteriocins is interesting, since they are a protein nature which show biopreservative potential. Its characteristics include antimicrobial action with specific action, not altering the intestinal microbiota, as they are digested by intestinal enzymes such as trypsin and pepsin, have a broad antimicrobial spectrum, acting in the inhibition of pathogenic bacteria and food spoilage (Zou, Jiang, Cheng, Fang & Huang 2018).

The aim of this study is the development of an active bioplastic film from a polymer blend, incorporated with a purified bacteriocin natural extract. The union of two polymers allows the synthesis of unique properties, acting in the improvement of specific flaws, originating from a single material. The novelty of this work is based on the establishment of the best proportion of the chitosan / agar-agar and plasticizer union in the solution for the synthesis of a bioplastic film, the use of antimicrobial extract of a new bacteriocin obtained from the food matrix, and the study in situ for feasibility of application as an active packaging.

2 MATERIALS AND METHODS

2.1 Materials

Chitosan (Oakwood Chemical) molar mass 170.7 - 198.5 kDa, degree of deacetylation 95% according to the manufacturer's data and Agar-agar (Himedia) were used as components forming the film. Glycerol (Alphatec) was used as plasticizer to improve matrix flexibility. For the chitosan dilution, acetic acid (Synth) 1M was used. Bacteriocin extract of *Lactobacillus sakei* obtained from food matrix, purified by precipitation of 80% ammonium sulphate, supernatant fraction was used as antibacterial agent. Agar Mueller Hinton (Himedia) was used for the analysis of disc-diffusion and microatmosphere.

2.2 Bioplastic film preparation

The filmogenic solution was obtained by adding agar-agar, chitosan and glycerol in the proportions established in the treatments (Table 1) of the central composite rotational design (CCRD, 2^3), according to Rodrigues & Iemma (2014). The experimental design aimed at identifying the influence of the formulation on film properties (TS: Tensile strength and EB:

elongation). The determined ranges were obtained from preliminary tests. Chitosan was dissolved in 1M acetic acid for 24h at 25 ° C and, subsequently, stirred for 30 min. The agar dissolved in distilled water. The film-forming solution (50 mL) was poured into a 15 cm diameter Petri dish. The films were obtained by the casting method, the components were solubilized with magnetic stirring in a heating plate at 80 ± 3 ° C (with the exception of chitosan) and evaporated in a convective dryer for 24 h at 40 ± 2 ° C. The films were conditioned in a desiccator with sulfuric acid solution, at a RH of 50% for 48 h before carrying out the analyzes.

Table 1 - Real and coded values of experimental design (%)

Level	Agar-agar(x1)	Chitosan (x2)	Glycerol (x3)
-1,68	0,0	0,0	10,0
-1	0,4	0,8	18,0
0	1,0	2,0	30,0
1	1,6	3,2	42,0
1,68	2,0	4,0	50,0

The percentage of plasticizer is expressed in relation to the total amount of polymer. The total film-forming solution was 50 mL.

The effect of the significant (independent) variables for TS and EB were identified through the Pareto Diagram, with 90 % confidence interval. The Analysis of Variance (ANOVA) and Fisher's Test were used to evaluate the significance and quality of the generated mathematical models. The optimization of the formulation was performed using the desirability function to maximize the bioplastics properties. The bioplastic films obtained in the optimized condition had the incorporation of purified bacteriocin extract (liquid form), in its formulation as a partial solvent substitute (10%), being this value defined in preliminary tests. All the analysis were done in triplicate and the result was expressed in mean and standard deviation.

2.3 Bacteriocin extract

The *Lactobacillus sakei* strain was previously isolated by the research group and cryopreserved at -14°C. The activation used *Man, Rogosa and Sharpe* broth (MRS) at a temperature of 32°C and agitation of 150 rpm for a time of 48 h. After the fermentation process, centrifugation was performed for 5500 rpm/15 min to remove the cells, since bacteriocins are extracellular compounds, thus obtaining the cell-free extract (Contessa et al., 2021).

The cell-free extract was purified by the ammonium sulfate precipitation method, where it was added under agitation to saturation of 0% to 80% of this same salt, where it remained

under refrigeration for 24 h, after this time of dormancy it was centrifuged at 5500 rpm/4°C for 15 min.

2.4 Bioplastic films characterization

2.4.1 Thickness

The film thickness was measured with a digital pachymeter (DIGIMESS 0.01mm) at fifteen random locations on the film.

2.4.2 Mechanical properties

The tensile strength at break (TS) and elongation at break (EB) were performed in texturometer (TA.XP2i,SMD,GBR) according to the *American Society for Testing and Materials* D 882 - 12 (ASTM, 2014) standard method. Samples were prepared with rectangular geometry 100 mm long and 25 mm wide and conditioned at 25°C in 50% relative humidity for 48 h prior to analysis. The initial grip separation was set at 50 mm and the speed at 50 min/mm. The measurements were performed three times, and the average was obtained. The calculation of tensile strength was found from Equation 1, followed by the calculation of elongation at break represented by Equation 2.

$$TS = FM/A \quad (1)$$

Where, *TS* is tensile strength (MPa), *FM* is the maximum force at the time of the film break (N) and A is Cross-sectional area (m^2).

$$EB = (DR/\ DI)\ x\ 100 \quad (2)$$

EB where represented by the elongation (%), *DR* by the distance at the moment of rupture (cm) and *DI* initial separation distance (cm).

2.4.3 Water vapor permeability

The permeability was determined gravimetrically by the standard method E96/E96M - 14 (ASTM, 2015), where the films were positioned in capsules containing anhydrous calcium chloride ($CaCl_2$). The set was conditioned in a chamber with a relative humidity of 50% being weighed after 7 days to determine the permeability of the films to water vapor from Equation 3.

$$wvp = (MP.L)/(t.A.\Delta P) \quad (3)$$

Where wvp is the permeability to water vapor (kg/Pa.s.m), *MP* is the mass of moisture absorbed (kg), L is the thickness of the film (m), t analysis time (s), A area of the exposed surface of the film (m²), ΔP is the water partial pressure difference through the film (Pa).

2.4.4 Water solubility

Samples of 2.5 cm in diameter of the films were used to determine solubility in water. Initially, the initial dry mass of the films was determined in a 105°C oven for 24 h. The samples were then immersed in 50 mL of distilled water and subjected to orbital agitation of 175 rpm for 24 h at a temperature of 25°C. After further drying the final dry mass was determined, thus the solubility of the films was expressed from Equation 4 (Riaz et al., 2018).

$$\text{SW} = ((MI - MF)/100)x100 \quad (4)$$

Where *SW* the (%) of solubility in water, *MI* is the initial dry mass (g) and *MF* is the final dried mass (g).

2.4.5 Swelling property

Films cut into samples of 2.5 x 2.5 cm were used as a specimen, which were weighed and immersed in miliQ (25°C) water for 2 min. The wet samples were cleaned with paper towels to absorb the excessive moisture and then weighed again. Measurements were repeated three times and an average was taken as a result. The amount of water absorbed was calculated using Equation 5 (Cao, Fu & He, 2007).

$$Swelling\ (\%) = \big(100(M2 - M1)\big)/M1 \quad (5)$$

Where *M1* and *M2* the masses (g) of the samples wet and dried, respectively.

2.4.6 Moisture content

The 2.5 x 2.5 cm specimens were weighed (m1) and dried at 105°C/24h, reweighed (m_2) and the moisture content (WC) determined (Equation 6) as the percentage of water lost during drying and reported on a wet basis (Cao, Fu & He, 2007). The measurements were repeated three times and an average was taken as a result.

$$WC\ (\%) = \big(100(M1 - M2)\big)/M1 \quad (6)$$

Where *M1* and *M2 are* the masses (g) of the samples wet and dried, respectively. Were repeated three times and an average was taken as a result.

2.4.7 Color, transmittance and opacity

The color of the films was determined by the colorimeter (Minolta® CR-300), using white as the standard. The values of (L*), (a*) and (b*) were used to characterize the color of the film on the Hunter Lab scale. The total color difference *(ΔE)* was calculated using Equation 7 *(*Riaz et al., 2020).

$$\Delta E = ((Li^* - L^*)^2 + (Ai^* - a^*)^2 + (Bi^* - b^*)^2)^{\frac{1}{2}} \quad (7)$$

Transmittance and opacity were determined from SP- 220 to 600nm spectrophotometer readings, the 1x4 cm long specimens were inserted in a glass bucket and the transmittance and absorbance readings were performed. The opacity was calculated using Equation 8 (Riaz et al., 2020).

$$O = (Abs_{600})/L \quad (8)$$

Where O is the opacity, Abs_{600} is the absorbance value at 600 nm, L is the thickness of the film (mm), Li^* refers to the brightness of the standard color (white) and L^* the luminosity of the sample, Ai^* indicates the red-green hue of the standard color and a^* the same shade as the sample, Bi^* represents the yellow-blue shade of the standard color and b^* of the sample.

2.4.8 Fourier Transform Infrared Spectroscopy (FTIR)

The film infrared spectra were characterized by Fourier Transform Infrared Spectroscopy (FT-IR) in spectrophotometer model FTIR-8400S (Shimadzu, Japan), combined with the ATR accessory.

2.4.9 Surface area and mean pore diameter

The adsorption technique (Quantachrome Instrument, NOVA 4200e, USA) and the BJH method (Barret, Joyner and Halenda) were used to obtain the characteristics surface area and mean pore diameter of biofilms. Initially the samples were kept at 77.35 K for 72 h in vacuum for degasification (Saibuatong; Phisalaphong, 2010).

2.4.10 Thermal stability

Thermal decomposition was evaluated by thermogravimetric analysis (TGA) using about 4.5 mg of sample in the instrument (SHIMADZU TGA 50, Japan) at a temperature range of 25 to 600°C with a heating rate of 10°C.min^{-1} and N_2 flow rate of 50 mL.min^{-1}.

2.4.11 *In vitro* antibacterial property

In order to evaluate the antibacterial properties, the adapted disc-diffusion method described by NCCLS (2003) was used for direct contact. Mueller Hinton Agar, inoculated with the indicator strains *Escherichia coli* (ATCC 11229), *Staphylococcus aureus* (ATCC 12598), *Listeria monocytogenes* (ATCC 7644) and *Salmonella enteritidis* (ATCC 13076) separately, was used as culture medium. Contamination occurred by spreading the standardized bacterial culture with approximately 1.5x10^8 CFU/mL on the surface (200µL). Contact was made using 6 mm diameter discs for film without extract (FSE), film with extract (FCE) and also 10µL of the purified supernatant fraction extract (EPS) of bacteriocin. The plates were refrigerated for 1 h in order to migrate the bacteriocin to the culture medium, similar to Meira, Zehetmeyer, Werner, & Brandelli. (2017), and incubated at 35°C/24 h. After the incubation period, the diameter of the inhibition zone was measured with a digital pachymeter.

For the microatmosphere analysis, the same media and indicator cultures were used, but the films were inserted in the lid of the Petri dish and these incubated inverted and wrapped with parafilm in order not to have detachment of vapors, thus providing a controlled microatmosphere (Higueras, López-Carballo, Gavara, & Hernández-Munoz, 2016).

2.4.12 Bioactive Release

The release profile was determined by immersion, (0.9 g of the films were submerged in 10 mL of 60% glycerol solution) for 20 days, being analyzed on the days (1, 2, 3, 4, 5, 10, 15, 20). The release was established from antibacterial analyses based on NCCLS (2003) methods, with SP- 220 to 625nm spectrophotometer readings, using standardized bacterial culture with approximately 1.5x10^8 CFU/mL of *Escherichia coli* (ATCC 11229) and *Staphylococcus aureus* (ATCC 12598). The 60% glycerol solution was used as a simulator of an aqueous food system with water activity between 0.7 to 1.0 (Chen, Xiao, Cai, & Liu, 2020; Xu, et al., 2019;).

2.5 Application *in situ*

To simulate the use of the film applied to food, the films with and without extract, were adhered to packages of cream cheese mines frescal, replacing the aluminum membrane traditionally used in this type of product. This way, thermotolerant coliforms and positive coagulase staphylococci were analyzed on the 7°, 14° and 21° accompanying day, in this time the product was stored inverted so that it would come into contact with the films, at cooling temperature.

An intentional contamination was also made to the cheese cream, with 10^6 CFU/of *Staphylococcus aureus* and *Escherichia coli*, separately. The microorganisms analyzed were thermotolerant coliforms and positive coagulase staphylococci performed by the traditional method of multiple tubes, in accordance with the *American Public Health Association* (APHA) and direct plate count method of APHA respectively (Brazil, 2001; Silva, 2010).

2.6 Statistical analysis

All data collected were presented with mean ± standard deviation and statistically analyzed by One-Way ANOVA and Tukey's later test with a 95% confidence level.

3 RESULTS AND DISCUSSION

3.1 Optimization of bioplastic formulation

The results obtained in the study of the formulation of bioplastic films through experimental design are presented in Table 2.

Table 2 – Matrix of the CCRD design (coded and real variables)

Treatments	Independent variable			Dependent variable	
	Agar-Agar (g)	Chitosan (g)	Glycerol (%m glycerol/m dry polymer mass)	TS (MPa)	EB (%)
1	-1 (0.20)	-1 (0.40)	-1 (18.09)	3.03±0.74	41.10±2.62
2	+1 (0.80)	-1 (0.40)	-1 (18.09)	10.36±0.94	25.60±2.29
3	-1 (0.20)	+1 (0.60)	-1 (18.09)	11.15±2.96	30.76±2.82
4	+1 (0.80)	+1 (0.60)	-1 (18.09)	6.79±2.86	24.46±3.48
5	-1 (0.20)	-1 (0.40)	+1 (41.09)	1.31±0.19	17.99±0.32
6	+1 (0.80)	-1 (0.40)	+1 (41.09)	5.63±1.56	17.45±2.65
7	-1 (0.20)	+1 (0.60)	+1 (41.09)	4.39±1.02	34.07±3.90
8	+1 (0.80)	+1 (0.60)	+1 (41.09)	7.09±1.03	25.23±3.20
9	-1.68 (0.00)	0 (1.00)	0 (30.00)	3.11±0.18	51.04±3.64
10	+1.68 (1.00)	0 (1.00)	0 (30.00)	9.47±2.53	14.90±2.20
11	0 (0.50)	-1.68 (0.00)	0 (30.00)	4.13±0.71	26.30±3.78
12	0 (0.50)	+1.68 (2.00)	0 (30.00)	4.57±1.56	17.88±2.77
13	0 (0.50)	0 (1.00)	-1.68 (10.00)	10.59±1.84	23.37±3.10
14	0 (0.50)	0 (1.00)	+1.68 (50.00)	3.54±0.69	18.18±3.91
15	0 (0.50)	0 (1.00)	0 (30.00)	8.88±1.04	22.95±1.83
16	0 (0.50)	0 (1.00)	0 (30.00)	8.08±2.09	22.16±3.21
17	0 (0.50)	0 (1.00)	0 (30.00)	10.467±0.28	21.00±1.68

TS: tensile strength at break; EB: elongation at break

From the results we can see the great variability of responses between treatments, indicating the influence of independent variables on the evaluated responses. The central point (treatment 15, 16 and 17) presented a small variation, showing the reproducibility of the process of obtaining the films. The values obtained for TS and EB varied between 1.31 to 11.15 MPa and 14.90 to 51.03%, respectively, and are in accordance with the ranges found by Andonegi et al. (2020) who studied chitosan-collagen blends with values around 11.03 MPa and 19.7% and Moradi et al. (2020) obtained rupture tension values between 8.73 to 14.11 MPa when

evaluating chitosan-polyethylene films. Jridi, Abdelhedi, Zouari, Fakhfakh, & Nasri, (2019), also obtained similar values for elongation at rupture when studying the interaction of compounds such as Arabic agar and gelatin-agar, with values from 7.59 to 27.25%.

Figure 1 indicates that for the tensile strength response the effect of the glycerol, agar-agar and chitosan variables was significant, indicating that with the increase of plasticizer the resistance is decreased. The same was observed by Chevalier, Assezat, Prochazka, & Oulahal, (2018) in which films with 13.2% glycerol had TS between 16 and 38 MPa and films with higher amount of plasticizer (24.2% glycerol) had TS between 2 and 13 MPa, which is due to the interaction between water and glycerol molecules, which lead to decreased intermolecular interactions, increasing the formation of vacant spaces and consequently decreased mechanical resistance (Sobral, Menegalli, Hubinger, & Roques, 2001). The higher the agar-agar proportion, the greater the tensile strength, due to the increase of the hydrogen bonds between the molecules, producing a more compact structure (Arham, Mulyati, Metusalach, & Salengke, 2016; Wu, Geng, Chang Yu, & Ma, 2009).

For the elongation at rupture the negative effect of the agar-agar variable was significant, indicating that when the agar-agar concentration increases the elongation is reduced. Wu, Geng, Chang, Yu, & Ma, (2009), when studying the effect of agar concentration in potato starch films, observed that the addition of agar agar up to 5% in relation to the amount of starch contributed to the increase in elongation, with higher concentrations of agar observed a decrease in elongation capacity. With lower concentrations, the agar allows the absorption of moisture, which decreases the hydrogen bonds, increasing the mobility of the film and increasing the elongation. However, at higher concentrations, agar acts in the formation of bonds between hydrogen and polymer, restricting the mobility of the polysaccharide chains and consequently an increase in elongation.

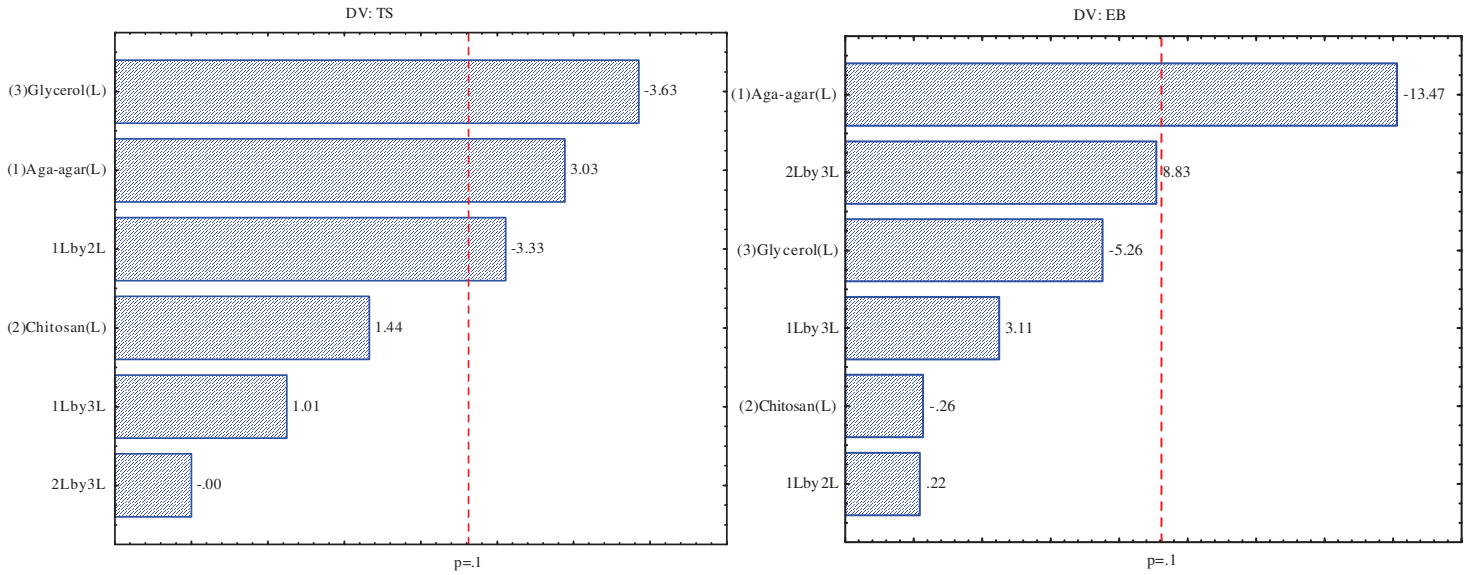


Figure 1 - Effect analysis for tensile strength (TS) and elongation at break (EB)

The analysis of variance (ANOVA) with a 90% significance level per residual SS was performed and the results are shown in Table 3.

Table 3 - Analysis of variation (ANOVA)

	SQ	**GL**	**MQ**	F_{cal}	F_{tab}	**R²**
			TS			
Regression	137.87	9	15.32	4.87	2.72	0.86
Residue	22.01	7	3.14			
Total	159.88	16				
			EB			
Regression	1116.53	9	124.06	3.22	2.78	0.80
Residue	269.81	7	38.544			
Total	1386.34	16				

It was possible to confirm the fit of the mathematical statistical models to the data obtained and their significance ($R^2 > 0.8$ and $F_{cal} > F_{tab}$) (Equations 7 and 8). The squares express the coded values of the model

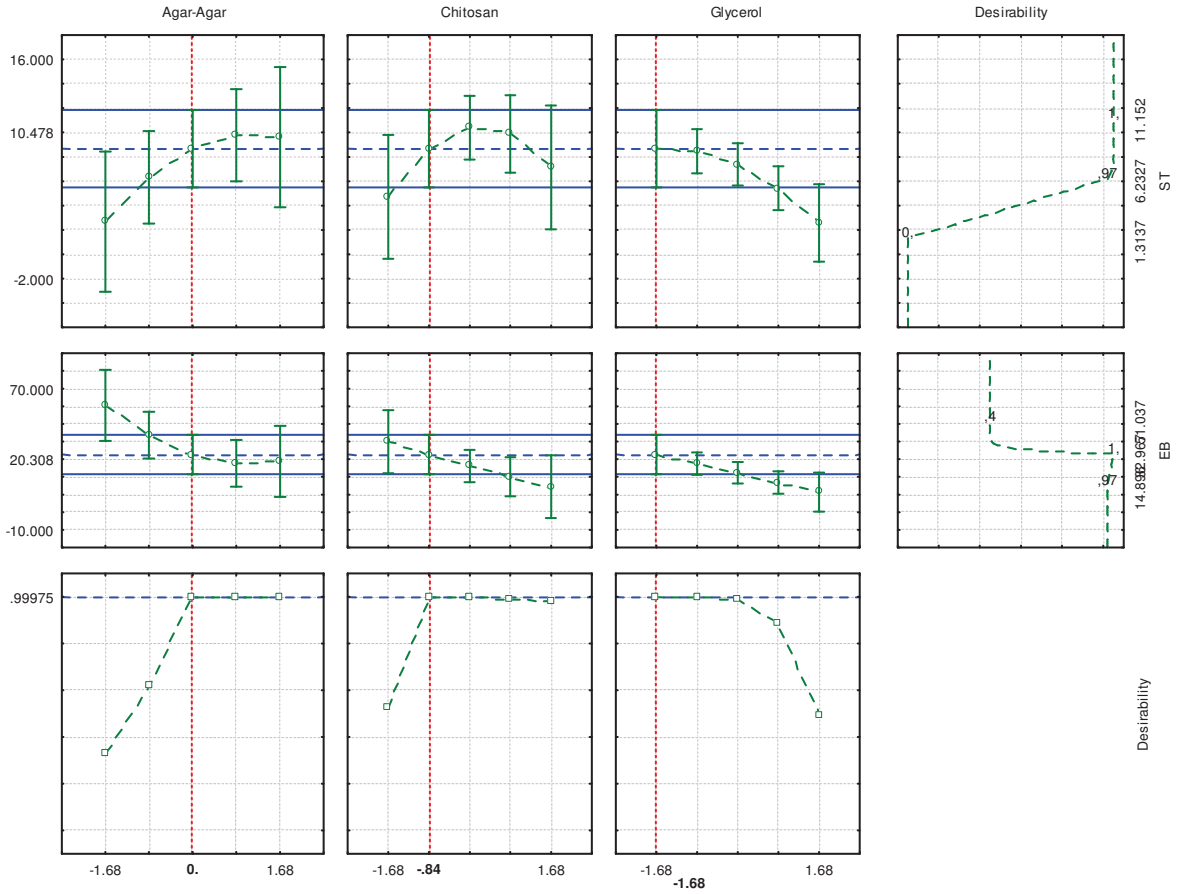
$$TS = 7.61 + 1.51(A) - 1.24(C)^2 - 1.81(G) - 1.66(A)(C) \quad (7)$$

$$EB = 22.22 - 6.73(A) + 4.16\,(A)^2 \quad (8)$$

Where A is Agar-Agar, *C* Chitosan and *G* Glycerol

The optimization of experimental conditions to obtain films with better responses, TS and EB was performed through the desirability analysis (Figure 2).

Figure 2 - Desirability function (TS and EB optimization)



According to the profile analysis, a desirability of 0.99 between a scale of 0 to 1 was obtained. The optimization considering the real variables as: 0.50 g, 0.49 g and 0.15 g for agar-agar, chitosan and glycerol, respectively, indicate the obtaining of films with 10.48 MPa of TS and 20.31% of EB. After playing the movie in the conditions indicated by the optimization and characterizing it the responses found were 13.57±2.17 MPa and 15.51±2.87 %, respectively. Both results can be considered similar to those predicted in the model, resulting in a bioplastic with the best characteristics according to the studied variables. The values found indicate good characteristics, being suitable for handling the packaging, allowing it not to break when wrapping the food and to remain intact during transport, packaging and handling.

3.2 Characterization of active bioplastic film

The results of the mechanical properties are illustrated in Table 4.

Table 4 - Tensile strength (TS) and elongation at break (EB) of films with FCE and without FSE extract

	TS (MPa)	**EB (%)**
ESF	13.57±2.17[a]	15.51±2.87[a]
FCE	11.08±2.33[a]	18.66±2.44[a]

The values are presented as mean ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$).

TS and EB did not present a significant difference ($p<0.05$) for films with and without extract, which indicates that the extract did not interfere significantly in the mechanical resistance of the film. According to Pastor, Sánchez-González, Chiralt, Cháfer & González-Martinéz, (2013), this property depends on the composition of the film, especially the concentration of polymers, which was not changed for the synthesis of ESF and FCE, which explains this behavior. This result indicates that there was possibly the interaction of all constituents used in the synthesis of bioplastics. Sánchez-González, Cháfer, González-Martínez, Chiralt, & Desobry, (2011) attributed that the addition of active compounds can provide structural discontinuities in the material due to the immiscibility of the constituents, leading to a decrease in intramolecular forces and consequently a reduction in mechanical properties. The results differ from Sánchez-González, González-Martínez, Chiralt, & Cháfer (2010), Riaz et al. (2018) and Crizel et al. (2018), who reported a reduction in mechanical properties of films added of other compounds (extracts) in relation to the control film.

The TS results of FCE and FSE films were slightly higher than those of Tan, Lim, Tay, Lee, & Thian, (2015). incorporated chitosan with 1.5% grapefruit seed extract, which showed tensile strength of 8.93 MPa. For the EB response, the films showed lower results than the green tea extract embedded agar-gelatin films, prepared by Giménez, Lacey, Santín, López-Caballero, & Montero (2013), where they obtained 59% elongation, however the handling of both was satisfactory.

Table 5 presents the results of physical, barrier and chemical characterization for films produced with and without extract.

Table 5 - Presents the results of physical. barrier and chemical characterization for films produced with and without extract.

	FSE	**FCE**
Thickness (mm)	0.048±0.009[b]	0.099±0.015[a]
WVP (10^{-13}kg.m^{-1}.Pa^{-1}.s^{-1})	1.498±0.97[a]	2.045±0.28[a]
Solubility (%)	10.281±1.038[b]	20.974±2.958[a]
Swelling property (%)	67.455±0.967[a]	35.755±3.245[b]
Moisture content (%)	20.936±0.325[a]	15.852±2.705[b]

Values are presented as mean ± standard deviation. Different letters on the same line indicate significant differences ($p < 0.05$).

The thickness of the films showed a significant difference between FCE and FSE control film due to the addition of solids present in the extract. Riaz et al. (2020), explain that thickness can also be changed due to the molecular interaction of polymers with the extract, and there may be short distance bonds, resulting in a more compact structure and consequently greater thickness. Similar findings were described by Kanmani & Rhim, (2014) in agar-based films incorporated with grapefruit seed extract, which obtained films in the range of 0.036 to 0.053 mm and Kaya et al (2018) in the range of 0.045 to 0.099 mm in chitosan films with fruit extract and *Berberis crataegina* seed oil.

The water vapor permeability (Table 5), did not present significant difference between FCE and FSE films. Both materials presented lower values than those reported by Atef, Rezaei & Behrooz (2014) and Wang et al. (2018) on agar-agar films reinforced with nanocrystalline and nanobacterial pulp, respectively, Hankar & Rhim (2016) on agar-agar and Reddy composite films; Rhim (2014) on agar and blackberry pulp nanocellulose films, which is a promising result. When water vapor permeability is favored in food products, they generally have a shorter lifespan due to the transfer between the external environment and the product, thus the use of bioplastic films aims at reducing this transfer.

The results for water solubility indicate a significant difference between films with and without extract. The film with extract had twice as solubility, possibly due to its hydrophilic characteristics, similar to what occurred with Narasagoudr, Hegde, Chougale, Masti & Dixit, (2020) in chitosan and poly (vinyl alcohol) films. However, the values were lower if compared to Zhao, Wei, Xu, & Han (2020) and Yadav, Mehrotra, Bhartiya, Singh & Dutta (2020) in gelatin and chitosan films and Wang & Rhim (2015) in agar-agar, alginate and collagen films,

which presented films with solubility around 43.45%, 58.13% and 63%, respectively. This is due to the presence of chitosan in the formulation, which has hydrophobic behavior. The solubility of the bioplastic film is important when the application in food is intended, especially when the food presents high content of humidity, which is the desired application in this study.

The property of swelling is the capacity of the film to retain water and the lower this capacity, the more effective the barrier of the food with the external environment. The values obtained indicate that there has been a significant decrease in the swelling property of the film containing the bacteriocin extract in relation to the control film. The values found in this study are below those found by Wang & Rhim (2015) and Cao, Fu & He (2007) in agar-agar films with 2363.7% and gelatin with 400 % respectively and similar to those found by Riaz et al. (2020) in chitosan films with Chinese chives root extract, where they also observed a reduction in the degree of swelling with the addition of the extract, from 57.38% to 40.49%, explained by the presence of hydroxyl (hydrophilic groups) in the chitosan molecule.

The visual characteristics of a package are attractive in the eyes of the consumer, which are often influenced by the color and transparency of the materials used (Riaz et al., 2020). The values of color, transmittance and opacity of the bioplastic films developed are presented in Table 6.

Table 6 – Measurement of color. transmittance and opacity

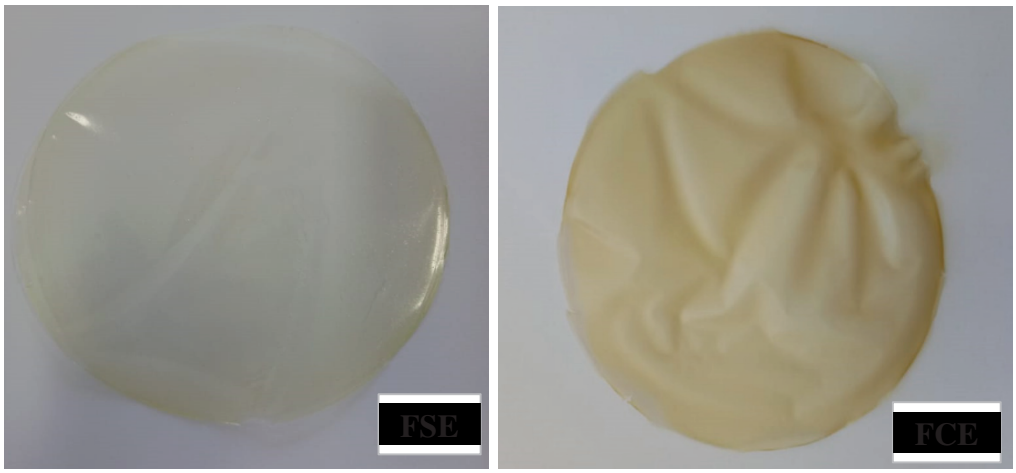
	L*	a*	b*	ΔE	Transmittance (%)	Opacity ($A.mm^{-1}$)
FSE	88.49 ± 0.19^{a}	0.60 ± 0.44^{b}	-1.78 ± 1.74^{b}	11.12 ± 0.26^{b}	86.37 ± 1.42^{a}	3.07 ± 0.59^{b}
FCE	81.12 ± 1.16^{b}	2.39 ± 0.16^{a}	11.96 ± 2.25^{a}	22.01 ± 2.21^{a}	37.57 ± 3.81^{b}	20.81 ± 1.27^{a}

Values are presented as mean ± standard deviation. Different letters on the same line indicate significant differences ($p<0.05$).

The control film is transparent and the addition of the extract has decreased the transparency and consequently increased the opacity, with a slightly yellowish color (Figure 3). The total color difference indicates greater color in the film with extract. The decrease of transmittance is a good characteristic when one intends an application as a barrier in the food area, since it protects the food from oxidation, loss of nutrients and discoloration (Arfat, Ahmed, Hiremath, Auras & Joseph, 2017). These observations are according to Rubilar, Candia, Cobos, Díaz & Pedreschi, (2016) in films of chitosan with nanoclay and LAE (ethyl Nα -dodecanoil-L- arginate), found a decrease in transmittance in relation to the control film, and differ from Haghighi et al. (2020), in films of chitosan and polyvinyl alcohol, which obtained only

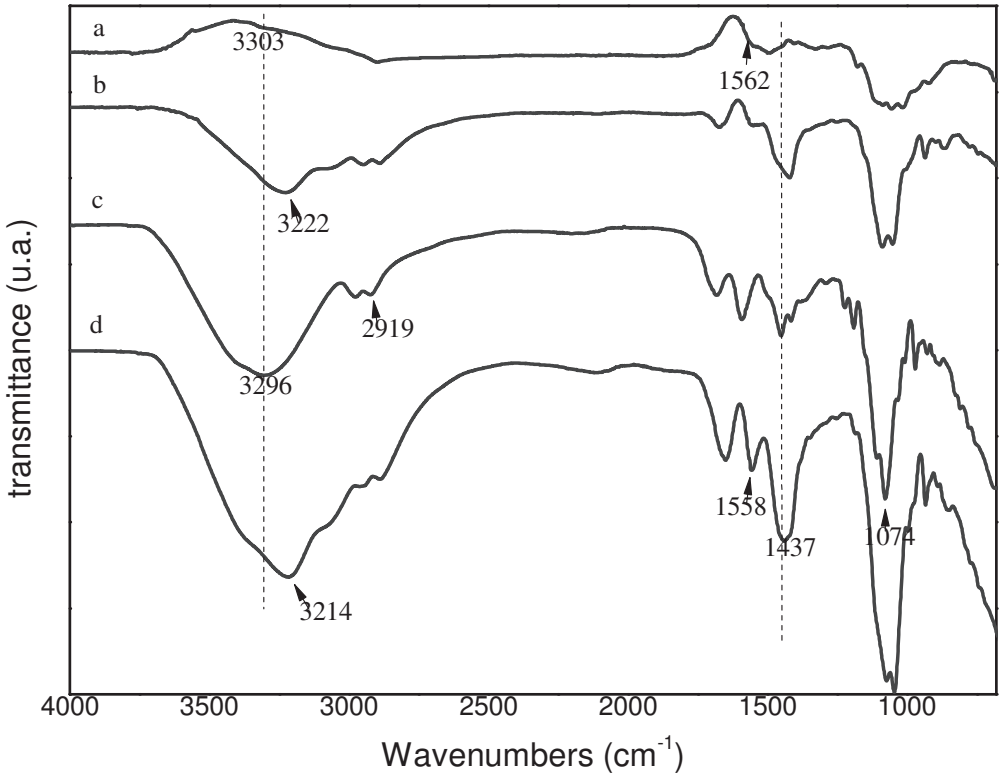
transparent films regardless of the presence or not of extract, because all showed opacity below 5. Adilah, Jamilah, Noranizan, & Hanani, (2018) in fish gelatin films incorporated of mango shell extract, also observed an increase in the opacity of the films as the extract was added, reported, however, opacity values below those found in this study, ranging from 0.06 to 1.11 A. mm^{-1}.

Figure 3 - Appearance of bioplastic films, without (FSE) and with extract (FCE).



The FTIR spectra of the film with and without extract, as well as the biopolymers (agar-agar and chitosan) were performed to determine the interactions of polymers and extract, and are shown in Figure 4.

Figure 4 - ATR-FTIR spectra of (a) chitosan polymer (b) agar-agar polymer (c) ESF (d) FCE



Peaks in the range 3210-3305 cm^{-1} indicate the presence of hydroxyl (OH), however a certain displacement between the samples is noticeable, indicating that there was an interaction of its constituents, caused by a small absorption displacement (Roy & Rhim, 2020). The peaks between 2900 - 2980 cm^{-1}, were verified in all samples and refers to grouping CH (Guerrero, Etxabide, Leceta, Penalba & Caba, 2014). The peaks around 1074 cm^{-1} present in all samples are due to the CO group (Rhim et al., 2014), where again the interaction of the compounds was noticed, because the biopolymers present lower peaks in this same wavelength, and the films without and with extract present much more intense peaks, being more pronounced in the FCE, demonstrating the interaction of this grouping. The peak 1558 cm^{-1} expressed in FCE and chitosan, corresponds to the NH connection (Antoniou et al., 2015), the other samples presented a peak very close to this, probably referring to the vibration of the ester grouping (COO^{-}) around the band of 1562 cm^{-1} (Roy, Shankar & Rhim, 2019). The 1437 cm^{-1} peak present in all samples

and of different intensities corresponds to the shear vibration of the CH grouping (Amalraj, Haponiuk, Thomas & Gopi, (2020); Rakmai, Cheirsilp, Mejuto, Torrado-Agrasar & Simal-Gándara, (2017).

The surface area, volume and pore size for the bioplastics samples with and without extract were estimated by the BJH method (Barret, Joyner and Halenda) and can be seen in Table 7.

Table 7 – BJH characterization of bioplastic films with and without extract (FSE) (FCE)

Bioplastics films	Surface area (m^2g^{-1})	Pore size (Å)
FSE	11.22	25.35
FCE	10.34	22.45

The film with extract showed a small reduction in surface area and pore sizes, due to the interaction of biopolymers with the extract, Zhong, Zhuang, Gu, & Zhao, (2019) observed that when there is an increase in the molecular weight of the solution, in this case caused by the addition of the extract, the film tends to become more compact and consequently less porous, however both samples were presented as microporous, according to Teixeira, Coutinho & Gomes (2001), sizes equal to or less than 200 Å, are thus classified. Saibuatong & Phisalaphong (2010) also obtained microporous films with a surface area of 15.7 m^2g^{-1}, synthesized from bacterial cellulose with the addition of 30% aloe vera.

The results of the thermogravimetric analysis can be seen in Figure 5, where it can be seen that both films presented three main stages of degradation, being the first stage of degradation between 40 and 45 °C attributed to water evaporation, with weight loss around 10% (Pavoni, Luchese & Tessaro, 2019). The second stage of degradation for the FCE at a temperature of 231.9 °C corresponds to the degradation of cellulose material (agar-agar) (Kumar, Boro, Ray, Mukherjee, & Dutta, 2019), the FSE presented a peak at 169 °C which refers to the degradation of plasticizer (glycerol) (Sadeghi & Shahedi, 2016), possibly the bacteriocin extract collaborated for a greater interaction of the plasticizer with the polymeric matrix, contributing to its greater thermal stability. The third stage refers to the degradation of chitosan, in a range of 252 - 294 °C (Hasan et al., 2020). It is also noted the similarity of the results for the control film compared to the added bacteriocin extract film, corroborating with the results of mechanical properties, surface analysis, water vapor permeability and FTIR.

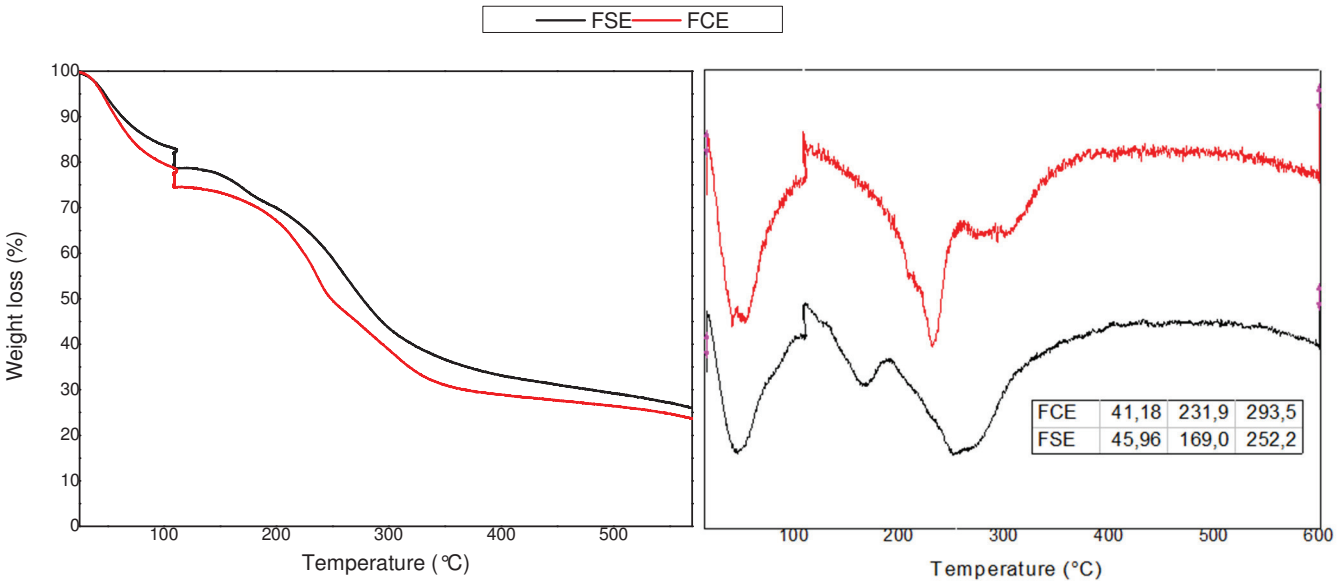
When the bioplastic film has an application for food product, it is interesting that it does not break down at the food packaging temperatures, since the degradation of the polymeric film

indicates the end of the food protection, once the film is undone. In this sense, the developed film presented good thermal characteristics, being degraded at high temperatures.

Qiao, Ma & Liu (2021) reported degradation of glycerol in chitosan films at 110 to 120°C, and in chitosan films with fungal extract around 140 to 210°C. The same authors observed degradation of chitosan at 266°C for chitosan control films and 257°C for chitosan films plus fungal extract.

Hankar & Rhim (2016) observed a thermal degradation around 300 to 370 °C in agar composite films and associated with agar degradation, since cellulosic materials have a degradation temperature in these neighborhoods. Reddy & Rhim (2014) report a wider range of thermal degradation for cellulosic materials, from 200 to 370°C, obtained on agar films and nanocellulose with papier-mâché pulp, a temperature range which was associated with agar degradation.

Figure 5 - (a) Mass loss (%) and (b) TGA curve for FCE and FSE



3.2.1 *In vitro* antibacterial analysis

The antibacterial effect of the purified bacteriocin extract and the embedded film extract can be seen in Table 8. The film without extract, only with the presence of biopolymers (chitosan and agar-agar) and glycerol showed no inhibition zone before the tested microbiota, which is in accordance with Hafsa et al. (2016), Ouattara, Simard, Piette, Bégin, & Holley, (2000) and Coma et al. (2002), explain that chitosan when in film form presents negligible antimicrobial properties, because it does not diffuse in the culture medium. In this way only the

organism in direct contact with its active sites is inhibited, because it is necessary that the amino grouping present in the chitosan, which is positively charged, enters in direct contact with anionic groups present in the surface of the cell of the microorganism, so that they can react and then inhibit the synthesis of new proteins.

Table 8 – Antibacterial effect (mm) of purified bacteriocin extract (EPS) and control bioplastic films (FSE) and incorporated bacteriocin (FCE) in front microbial tests

	Escherichia coli	*Salmonella enteritidis*	*Staphylococcus aureus*	*Listeria monocytogenes*
EPS	13.04 ± 1.76^{aA}	11.38 ± 0.57^{aAB}	9.27 ± 0.015^{bB}	-
FCE	13.57 ± 0.74^{aA}	11.80 ± 1.39^{aAB}	19.86 ± 0.43^{aC}	9.91 ± 0.05^{B}
FSE	No inhibition zone			

The values are presented as mean ± standard deviation. Different lower case letters in the same column indicate significant differences. different upper case letters in the same line indicate significant differences ($p < 0.05$).

The absence of bacterial growth around the films or extract is characterized by a clear zone, visible to the naked eye, and called a halo of inhibition. When the microorganisms *Escherichia coli* and *Salmonella enteritidis were* analyzed, there was no significant difference ($p<0.05$) between the bacteriocin extract and the film incorporated with it. For *Staphylococcus aureus*, the film presented a greater halo of inhibition than the extract, indicating that possibly the extract has been potentiated when entering in contact with the chitosan, showing itself more efficient in front of microorganisms of Gram-positive membrane. The results indicate that the bacteriocin extract has antibacterial action when applied to the bioplastic film, since it maintained or increased the inhibition halos, thus confirming the efficiency of the bioplastic film developed in this study and its potential to be applied as active packaging in a food. Similar findings were reported by Sugumar, Mukherjee & Chandrasekaran (2005) found inhibition halos of 7, 11 and 15 mm for chitosan films with different concentrations of *eucalyptus* oil nanoemulsion against *Staphylococcus aureus*. Wu et al. (2019), reported inhibition halos between 6 and 9 mm for *Escherichia coli* and 8 to 19 mm for *Staphylococcus aureus* in chitosan nanocomposites and Ɛ-polylisin.

With the concern of real efficiency in the application as active packaging, the microatmosphere test (Figure 6) indicates that the packaging does not need to be in direct contact with the food to be effective, since the active compound has volatility and can act, even if not in direct contact.

Figure 6 – Micro atmosphere test in front of *Escherichia coli*

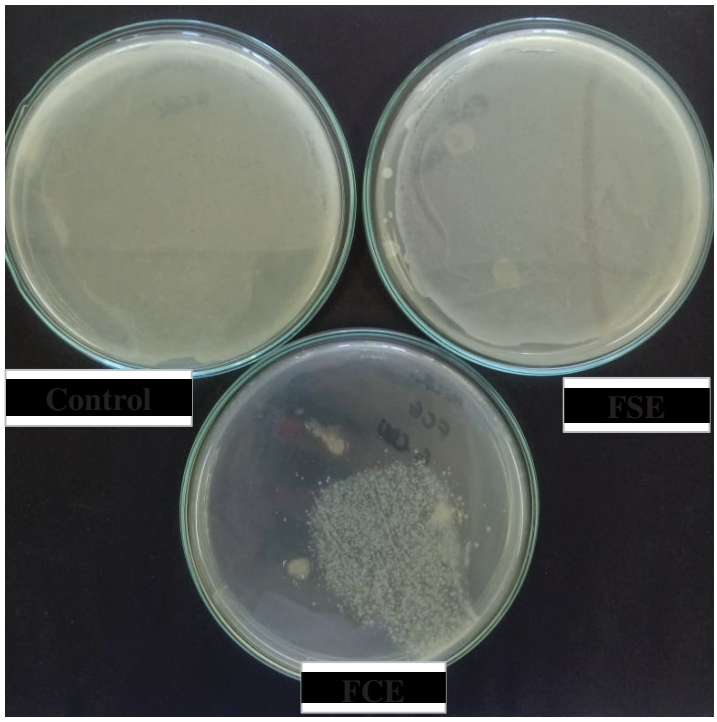


Figure 6 indicates the development of *E.coli,* inoculated in the *petri dish* (control), and two more *petri* dishes where the *E.coli* culture was sown, being the one called FSE, which contains the film without the incorporation of the extract and the one containing the film with the incorporation of the bacteriocin extract (FCE). It is noted that both in the control and in the ESF there is a clouding of the culture medium, indicating the proliferation of *E.coli,* after 24 h of incubation, while in the FCE small colonies can be seen in a region of the plaque, suggesting that the volatilization of the compound occurred and was efficient in inhibiting microbial development. With this technique the inhibitory action is due to the volatile compounds of the extract, which in the vapor phase come into contact with microorganisms (Goñi et al., 2009). The results are in accordance with those of Kashiri et al. (2017), which obtained positive effects for the micro atmosphere test for *Listeria monocytogenes* and *Escherichia coli*, in zeine films with essential oil of *Zataria multiflora* Boiss, however a greater concentration of the extracts used was necessary in comparison with disc diffusion analysis under the same conditions. Dannenberg et al., (2017) obtained satisfactory results in microbial reduction of *Salmonella Typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes* when using pink pepper essential oil as an antimicrobial in cellulose acetate film.

Thus, the developed film has potential for application in a food product, as it has adequate mechanical characteristics for handling and packaging, has excellent physical and barrier properties, antibacterial capacity, and can act both in direct contact and by microatmosphere, thus helping in increasing the product's lifespan.

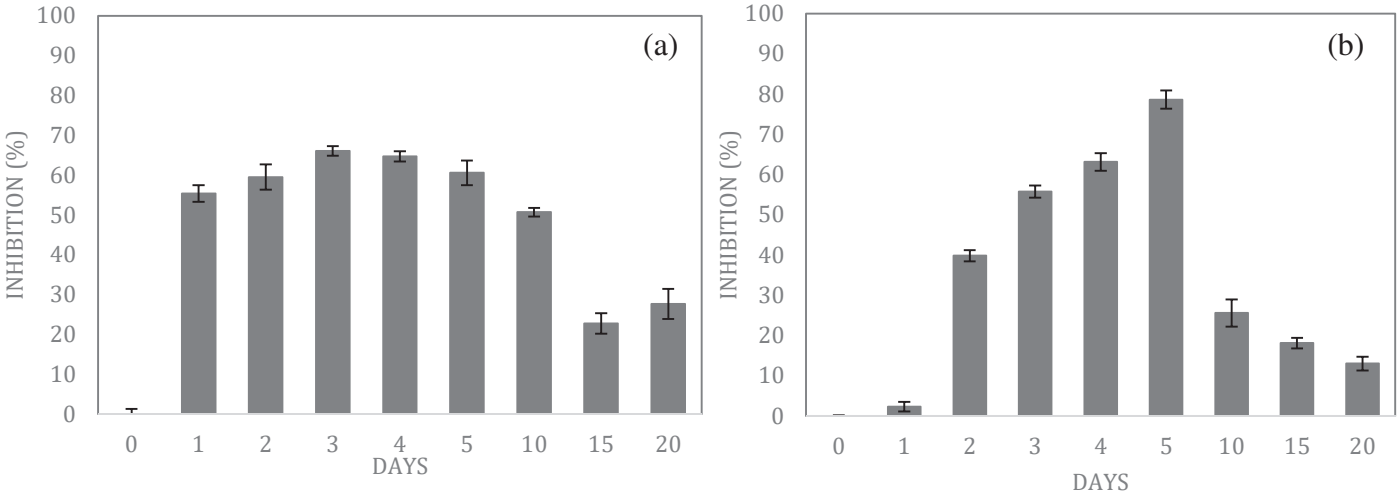
3.2.2 Release profile

The release profile of the bacteriocin extract incorporated in the bioplastic film (aga-agar/chitosan) is illustrated in Figure 7, where it is noted that bacteriocin was gradually released, reaching the maximum release between the third and fifth day of contact with the simulant solution, where it presented 66.07±1.2% of inhibition against *Escherichia coli* on the third day and 78.61±2.3 under *Staphylococcus aureus* on the fifth day. The release profile was traced from the activity of the compound and not the compound itself, in this way as the test microbiota is composed of different microorganisms, one representative of the group of Gram-positive and another of Gram-negative, the performance bacteriocin occurs differently due to the divergence of cell walls.

The gradual release is made possible by the characteristics of the polymeric matrix (Rahman, Ramanathan & Sankar, 2014) mainly by solubility and porosity, which was favored in this study, since the film presents a low solubility (20.97%) and a microporous surface analysis (22.45Å). The release of bacteriocin extract provides microbial inhibition, so the profile was elaborated in front of the inhibition that occurred in front of the microbiota test, where it is noted that during all the time of analysis the microbial reproduction was reduced in comparison with the control sample (time 0), which presented 100% of microbial growth and in turn 0% of inhibition.

The same analysis was performed with the film without extract (FSE), and showed no signs of inhibition, indicating that the inhibition observed in the FCE comes from the bacteriocin extract added to the formulation.

Figure 7 - Bacteriocin release incorporated in the polymeric matrix (a) inhibition under *Escherichia coli* (b) inhibition under *Staphylococcus aureus*



Roy & Rhim (2020), found that in chitosan and curcumin films, curcumin is released in up to 100 min and slows down to the point of balance with the solution. Xu et al. (2019), when analyzing films of gum arabic and chitosan incorporated of essential oils of cinnamon and clove, found an initial fast release that remained constant during 600 min, in 40 min there was a release of 58.90% of the oils and 99.42% in 300 min of contact with the same simulant solution analyzed in this study. Chen, Xiao, Cai, & Liu, (2020), observed a release of 60% of the tea polyphenol in 150 min of contact with the simulant solution and was kept constant for 600 min, when analyzing zein and gelatin films with polyphenol.

3.5 Microbiological monitoring *in situ*

The results of the microbiological follow-up of the minas frescal cream cheese are shown in Table 9.

Table 9 - Microbiological stability *in situ*

Coagulase positive staphylococci (CFU/g)		
Analysis days	FSE	FCE
Time 0	$1.1x10^{6}±1.65x10^{5A}$	
7th Day	$8.1x10^{5}±1.4x10^{5aB}$	$2.8x10^{5}±2.4x10^{5bB}$
14th Day	$4.3x10^{4}±1.5x10^{4aC}$	$1.1x10^{4}±1.0x10^{3bC}$
21th Day	$1.8x10^{4}±1.3x10^{4aD}$	$3.7x10^{3}±4.36x10^{2aD}$
Thermotolerant coliforms (NPM/g)		
Time 0	$150±43.3^{A}$	
7th Day	$93±0.0^{aB}$	$43±2.89^{bB}$
14th Day	$75±16.26^{aC}$	$43±0.00^{bB}$
21th Day	$43±0.00^{aD}$	$23±0.00^{bC}$

The values are presented as mean ± standard deviation. Different lower case letters in the same column indicate significant differences, different upper case letters in the same line indicate significant differences ($p < 0.05$).

It is observed that after the storage a reduction in the count was obtained, including the control film, due to injuries caused by the cold, which is predicted because the cold acts inhibiting or delaying the multiplication of microorganisms (Jay, 2005). However, the film with extract (FCE) contributed to a greater reduction in the microbial load, since it showed a 53.4% reduction compared to the FSE in the same storage time. The FCE reduced 2.62 log CFU/g while the ESF reduced 1.79 log CFU/g.

Seydim, Sarikus-Tutal & Sogut (2020) in 15 days storage of Kasar cheeses using whey protein films with nisin as a separator obtained a log reduction for *Escherichia coli* and *Staphylococcus aureus*. Artiga-artigas, Acevedo-Fani & Martín-Belloso (2017) also found a decrease from 6 to 4.6 log CFU/g in the count of *Staphylococcus aureus* during 15 days of storage of cheese samples coated with edible nanoemulsion-based essential oil of oregano and tangerine fiber.

Dannenberg et al. (2017), when analyzing the antibacterial action of cellulose acetate films incorporated with pink pepper essential oil in sliced mozzarella cheese, obtained a reduction from 4.30 to 2.91 log CFU/g for *Staphylococcus aureus in* 12 days of storage and for *Escherichia coli* found no significant action. Goksen,, Fabra, Ekiz, & López-Rubio, (2020) found a reduction of 1.7 and 1.6 log CFU/g in the growth of *Staphylococcus aureus* by adding zein film with essential oil extracted from *Laurus nobilis (*bay leaf) and *Rosmarinus officinalis* (rosemary) respectively, in slices of guoda cheese for a time of 28 days. Youssef, El-Sayed, El-Sayed, Salama & Dufresne, (2016), found a reduction of 1.30 log CFU/g in the coliform group

over a period of 30 days in Egyptian soft white cheese when using bionanocomposite film of chitosan/carboxymethyl cellulose/zinc oxide.

In view of what has been reported, the importance of using this type of active packaging is noted, in order to reduce the incidence of these high contaminations. It can be seen that the bacteriocin embedded film studied in the present work has reduced from $1.1x10^6 \pm 1.65x10^5$ CFU/g for $3.7x10^3 \pm 4.36x10^2$ for *Staphylococcus aureus* and 150±43.3 NPM/g for 23±0.00 NPM/g for *Escherichia coli*, presenting itself as a great alternative for use in products of this origin.

4 CONCLUSION

From the analysis of the effects, the influence of independent variables (glycerol, agar-agar, chitosan and the interaction of agar-agar and chitosan) for tensile strength and (agar-agar and the interaction of chitosan and glycerol) for elongation at rupture was verified. The desirability function allowed the optimization of concentrations for synthesis of bioplastic films. With the addition of the extract, statistically there was no significant difference in the mechanical properties of the films and water permeability. The solubility increased with the increase of the extract that has hydrophilic characteristics, from 10.28±1.03 (%) to 20.97±2.95 (%), however it still presented a low solubility when compared with the literature, not being an interference when adding in a food product with high free water content. The swelling property was improved from 67.45±455 (%) to 35.75±3.24 (%), as well as the opacity which had an increase from 3.07±0.59 ($A.mm^{-1}$) to 20.81±1.27 ($A.mm^{-1}$), thus avoiding the incidence of UV light directly on the product. Infrared spectroscopy, surface analysis and thermogravimetric analysis found that there was an interaction of all the constituents of the filmogenic solution. The release profile of the active compound found a gradual release during the time of contact with the simulant solution, having between the third and the fifth day the release peaks, allowing, however, the reduction of microbial development present throughout the time analyzed. The antibacterial responses *in vitro*, *in situ* and microatmosphere showed the efficacy of the active film against the microbiota test, presenting a reduction of 2.62 log CFU/g directly in the food. It is concluded that the film has active capacity with characteristics that favor the performance for application in a food product, as it has adequate mechanical characteristics for handling and packaging, has excellent physical and barrier properties, antibacterial capacity, and can act both in direct contact as well as by microatmosphere, thus helping to increase the product's lifespan.

ACKNOWLEDGEMENTS

The present work was carried out with the support of the Coordination of Improvement of Higher Level Personnel - Brazil (CAPES) - Financing Code 001.

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CAPÍTULO 5

Conclusão

Conclui-se que a síntese do filme bioplástico foi bem-sucedida, atendendo as características adequadas para conter, proteger e aumentar a vida útil do creme de queijo minas frescal. As características mecânicas dos filmes foram adequadas para o manuseio, como resistência à tração e alongamento na ruptura, apresentando baixa permeabilidade ao vapor de água e propriedade de intumescimento, devido a aplicação requerida. O filme permaneceu intacto ao entrar em contato com o alimento, permitindo desta forma uma barreira eficiente. Ainda, apresentou uma baixa transmitância, permitindo desta forma uma barreira a incidência direta de luz a fim de manter as características sensoriais do produto. Houve homogeneidade dos compostos formadores da matriz. As respostas antibacterianas *in vitro*, *in situ* e de microatmosfera evidenciaram a eficácia do filme ativo. O filme bioplástico permitiu uma liberação gradual do composto bioativo durante o tempo de contato, permitindo o aumento da estabilidade microbiológica do produto, reduzindo 2,62log UFC/g diretamente no alimento. Desta forma o filme bioplástico apresentou potencialidade para ser utilizado como embalagem ativa.

CAPÍTULO 6

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